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Article

A New 5α,8α-Epidioxysterol from the Soft Coral *Sinularia gaweli*

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Abstract: A new sterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methyl-cholest-6,9(11)-dien-3β-ol (**1**), and two known sterols, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-

22,23-methylene-24-methylcholest-6-en-3β-ol (**2**) and 24-methylenecholestane-1α,3β,5α, 6β,11α-pentol (**3**), were isolated from the soft coral *Sinularia gaweli*. The structure of sterol **1** was established by spectroscopic methods and by comparison of the spectral data with those of known analogues. The cytotoxicity of sterols **1**–**3** towards various tumor cells is reported.

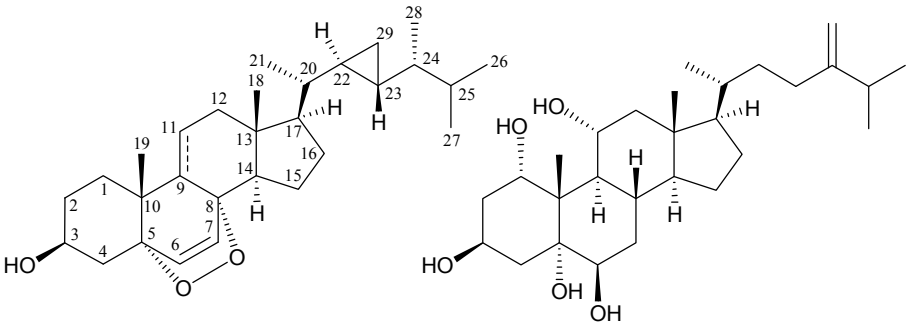
Keywords: *Sinularia*; epidioxysterol; cytotoxicity

1. Introduction

Soft corals belonging to the genus *Sinularia* have been well-recognized as marine organisms containing various natural products that show interesting bioactivities [1–3]. A series of cytotoxic [4–12], anti-inflammatory [7,11–13] and antiviral [10] steroids have been isolated from *Sinularia* sp. octocorals collected off the waters of Taiwan. In continuation with our search for new natural substances, the organic extract of soft coral *Sinularia gaweli* (Figure 1) was studied, which displayed meaningful signals in NMR studies. Previous investigations of the chemical constituents of *S. gaweli* yielded two norcembranoidal diterpenes, 5-episinuleptolide acetate and scabrolide D [14]. In further studies of *S. gaweli*, a new sterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6,9(11)-dien-3β-ol (**1**), and two known sterols, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol (**2**) [4] and 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**3**) [15,16], were isolated (Figure 1).

Figure 1. The soft coral *Sinularia gaweli* and the structures of (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6,9(11)-dien-3β-ol (**1**), (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol (**2**) and 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**3**).

Sinularia gaweli



1: $\Delta^{9,11}$, **2**: 9,11-saturated **3**

2. Results and Discussion

(22*R*,23*R*,24*R*)-5α,8α-Epidioxy-22,23-methylene-24-methylcholest-6,9(11)-dien-3β-ol (**1**) was isolated as a white powder. The molecular formula of **1** was established as $C_{29}H_{44}O_3$ (eight degrees of unsaturation) from a $[M+Na]^+$ molecule at *m/z* 463.3192 in HRESIMS (calcd for $C_{29}H_{44}O_3Na$,

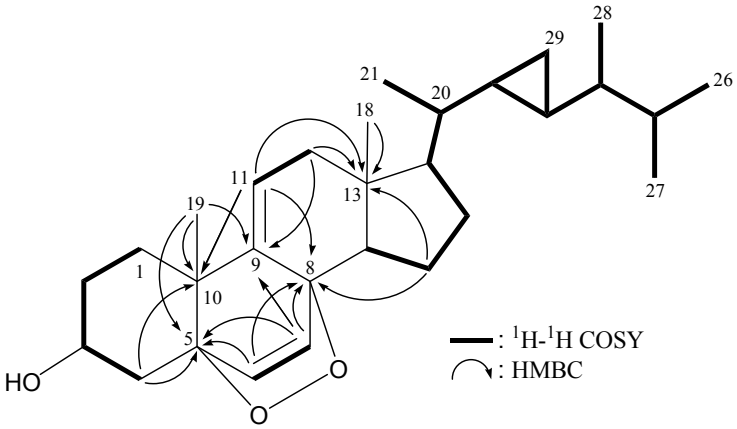
463.3188). The ^{13}C-NMR and DEPT spectra of **1** showed this compound to have 29 carbons (Table 1), including six methyls, seven sp^3 methylenes, eight sp^3 methines, three sp^2 methines, four sp^3 quaternary carbons and an sp^2 quaternary carbon. From the NMR spectra (Table 1), the presence of three oxygenated C atoms at δ_C 82.7 (C-5), 78.4 (C-8) and 66.3 (CH-3) in the ^{13}C-NMR spectrum and an oxymethine proton at δ_H 4.02 (1H, m, H-3) in the ^{1}H-NMR spectrum was determined. This sterol was further recognized as a 5α,8α-epidioxysterol by the presence of the characteristic signals for H-6 (δ_H 6.60, J = 8.0 Hz) and H-7 (δ_H 6.28, J = 8.0 Hz) in the ^{1}H-NMR spectrum [4,17]. Four protons appeared at δ_H 0.14 (2H, m, H_2-29), 0.33 (1H, m, H-23) and 0.55 (1H, m, H-22), indicating the presence of a cyclopropyl moiety in **1**. Two singlets, which appeared at δ_H 0.68 (3H) and 1.09 (3H), were attributed to Me-18 and Me-19, respectively. Four doublets at δ_H 0.91 (3H, J = 6.4 Hz), 0.86 (3H, J = 6.8 Hz), 0.89 (3H, J = 6.8 Hz) and 0.92 (3H, J = 6.4 Hz) were due to the presence of Me-21, Me-26, Me-27 and Me-28, respectively. The above data suggested that **1** is a peroxysteroid containing a 22,23-methylene-24-methyl moiety in the side chain.

Table 1. ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data, ^{1}H–^{1}H COSY and HMBC correlations for sterol **1**.

Position	δ_H (J in Hz)	δ_C, Mult.	^{1}H–^{1}H COSY	HMBC (H→C)
1	2.11 m; 1.70 m	32.6, CH_2	H_2-2	n.o.
2	1.91 m; 1.55 m	30.6, CH_2	H_2-1, H-3	C-3
3	4.02 m	66.3, CH	H_2-2, H_2-4	n.o.
4	2.14 dd (13.6, 2.0); 1.92 dd (13.6, 11.6)	36.1, CH_2	H-3	C-2, -3, -5, -10
5		82.7, C		
6	6.60 d (8.0)	130.8, CH	H-7	C-4, -5, -8
7	6.28 d (8.0)	135.4, CH	H-6	C-5, -8, -9, -14
8		78.4, C		
9		142.5, C		
10		37.9, C		
11	5.42 dd (6.0, 2.0)	119.8, CH	H_2-12	C-8, -10, -12, -13
12	2.28 dd (16.8, 6.0); 2.09 dd (16.8, 2.0)	41.2, CH_2	H-11	C-9, -11, -13, -14, -17
13		44.1, C		
14	1.83 dd (12.0, 8.0)	47.8, CH	H_2-15	C-12, -15
15	1.75 m; 1.61 m	21.2, CH_2	H-14, H_2-16	C-8, -13, -16
16	2.20 m	28.4, CH_2	H_2-15, H-17	n.o.
17	1.49 m	57.4, CH	H_2-16, H-20	n.o.
18	0.68 s	12.6, CH_3		C-12, -13, -14, -17
19	1.09 s	25.5, CH_3		C-1, -5, -9, -10
20	0.88 m	39.7, CH	H-17, H_3-21, H-22	C-17
21	0.91 d (6.4)	19.0, CH_3	H-20	C-20, -22
22	0.56 m	24.2, CH	H-20, H-23, H_2-29	n.o.
23	0.33 m	25.1, CH	H-22, H-24, H_2-29	n.o.
24	0.55 m	44.9, CH	H-23, H-25, H_3-28	n.o.
25	1.64 m	32.8, CH	H-24, H_3-26, H_3-27	C-24
26	0.86 d (6.8)	18.5, CH_3	H-25	C-24, -25, -27
27	0.89 d (6.8)	20.7, CH_3	H-25	C-24, -25, -26
28	0.92 d (6.4)	15.8, CH_3	H-24	C-24, -25
29	0.14 m	10.5, CH_2	H-22, H-23	C-20, -22, -24

From the ^{1}H–^{1}H COSY spectrum, several structural units, including H_2-1/H_2-2/H-3/H_2-4, H-6/H-7, H-11/H_2-12, H-14/H_2-15/H_2-16/H-17/H-20/H-22/H-23/H-24/H-25/H_3-26(H_3-27), H-20/H_3-21, H-22/H_2-29, H-23/H_2-29 and H-24/H_3-28, were identified (Table 1 and Figure 2). These data, together with the key HMBC correlations between protons and quaternary carbons, such as H_2-4, H-6, H-7, H_3-19/C-5; H-6, H-7, H-11, H_2-15/C-8; H-7, H_2-12, H_3-19/C-9; H_2-4, H-11, H_3-19/C-10; and H-11, H_2-12, H_2-15, H_3-18/C-13, permitted the elucidation of the main carbon skeleton of 1 (Table 1 and Figure 2). The ring junctions C-18 and C-19 methyl groups were positioned at C-13 and C-10 from the HMBC correlations between H_3-18/C-12, -13, -14, -17 and H_3-19/C-1, -5, -9, -10. An oxymethine unit at δ_C 66.3 correlated to the methine proton at δ_H 4.02 in the HMQC spectrum, proving the attachment of a hydroxy group at C-3.

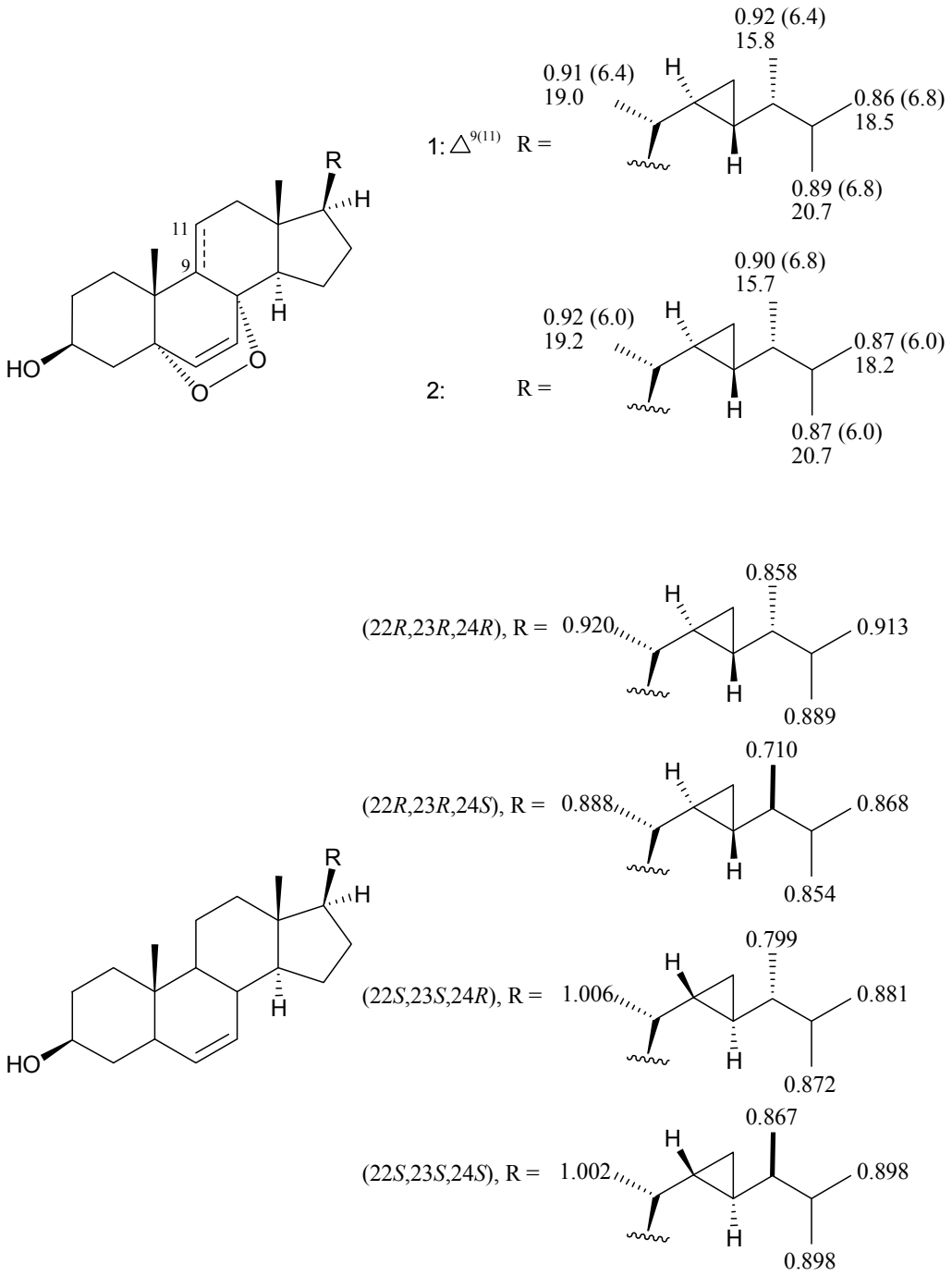
Figure 2. The ^{1}H–^{1}H COSY and selective HMBC correlations (protons→quaternary carbons) for sterol **1**.



Because of the signals for protons H-22/H-24 and H-20/H_3-21, H_3-26, H_3-27 are overlapped in the ^{1}H spectrum of **1**, it is difficult to judge the relative configuration of the cyclopropyl moiety by their NOE effect in the NOESY spectrum. However, by comparison of the ^{1}H- and ^{13}C-NMR chemical shifts of Me-21, Me-26, Me-27 and Me-28 with those of a known epidioxysterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol (**2**) [4,18] and four synthetic demethyl-gorgosterol isomers [19] (Figure 3), it was suggested that the stereochemistry of **1** at the side chain should be assigned as 22*R*, 23*R* and 24*R*, as per those of **2**. The assignment of the carbon shifts of **1** was based on the comparison of these data with those of the tetracyclic system of **2** [4]. In the HMQC spectrum of **1**, the doublet methyls appearing at δ_H 0.86 (J = 6.8 Hz, H_3-26) and 0.92 (J = 6.4 Hz, H_3-28) showed ^1J-correlations with δ_C 18.5 and 15.8, respectively; and the methine protons appearing at δ_H 0.33 (m, H-23) and 0.56 (m, H-22) showed ^1J-correlations with δ_C 25.1 and 24.2, respectively. We suggest that the partial ^{1}H and ^{13}C-NMR chemical shifts for the side chain of steroid **2** that were reported previously should be re-examined [4,20]. Based on the above findings, the structure of **1** was tentatively established as (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6,9 (11)-dien-3β-ol.

In previous studies, the 5α,8α-epidioxy sterols were supposed to have arisen from $\Delta^{5,7}$-sterols by photooxidization during storage and/or chromatographic separation [21–23] with a self-perpetuating mechanism [23]. $\Delta^{5,7}$-Sterol analogues were not obtained from *S. gaweli*; at this point it is difficult to infer whether epidioxysterol **1** from *S. gaweli* is a natural product or an artifact.

Figure 3. The ^{1}H and ^{13}C-NMR chemical shifts of the side-chain methyl groups of epidioxysterols **1** and **2** and synthetic isomers of demethylgorgosterols [4,18,19].



Sterols **2** and **3** were identified as (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol and 24-methylenecholestane-1α,3β,5α,6β,11α-pentol, which have been previously isolated from a Formosan soft coral *Sinularia* sp. [4] and an Andaman Sea soft coral *Sinularia dissecta* [15,16], respectively. Their spectral data were in full agreement with those of previously reported.

The cytotoxicity of sterols **1**–**3** towards K562 (human erythromyeloblastoid leukemia), MOLT-4 (human acute lymphoblastic leukemia) and HL-60 (human promyelocytic leukemia) cells was studied, and the results are shown in Table 2. These data showed that sterol **3** exhibited significant cytotoxicity towards HL-60 cells.

Table 2. Cytotoxic data of sterols **1**–**3**.

Compounds	Cell lines IC_{50} (μg/mL)		
	K562	MOLT-4	HL-60
1	NA	15.70	NA
2	NA	NA	12.14
3	9.71	6.91	3.39
Doxorubicin [a]	0.20	0.01	0.03

[a] Doxorubicin was used as the positive control. NA = not active at 20 μg/mL for 72 h.

3. Experimental

3.1. General Procedures

Optical rotation values were measured with a Jasco-P1010 digital polarimeter. Infrared spectra were obtained on a Varian Diglab FTS 1000 FT-IR spectrophotometer. NMR spectra were recorded on a Varian Mercury Plus 400 FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ or C_5D_5N at 25 °C. ESIMS and HRESIMS data were recorded on a Bruker APEX II mass spectrometer. Column chromatography was performed on silica gel (230–400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm, Merck) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. Normal phase HPLC (NP-HPLC) was performed using a system comprised of a Hitachi L-7110 pump, a Hitachi L-7455 photodiode array detector and a Rheodyne 7725 injection port. A normal phase column (Supelco Ascentis® Si Cat #:581515-U, 25 cm × 21.2 mm, 5 μm) was used for NP-HPLC. Reverse phase HPLC (RP-HPLC) was performed using a system comprised of a Hitachi L-7100 pump, a Hitachi L-2455 photodiode array detector and a Rheodyne 7725 injection port. A reverse phase column (Varian Polaris C18-A, 250 mm × 10 mm, 5 μm) was used for RP-HPLC.

3.2. Animal Material

Specimens of the soft coral *Sinularia gaweli* were collected by hand using scuba equipment off the coast of Sansiantai, Taitung County, Taiwan on Oct. 13, 2011, and stored in a freezer (−20 °C) until extraction. This organism was identified by comparison with previous descriptions [24]. A voucher specimen (NMMBA-TWSC-11007) was deposited in the National Museum of Marine Biology and Aquarium, Taiwan.

3.3. Extraction and Isolation

The freeze-dried and minced material of *Sinularia gaweli* (wet weight 1.30 kg, dry weight 328 g) was extracted with ethyl acetate (EtOAc) at 25 °C (2 L × 10). The EtOAc extract left after removal of the solvent (11.4 g) was separated by silica gel and eluted using *n*-hexane/EtOAc/acetone in a stepwise fashion to yield 14 fractions A–N. Fraction F was separated by NP-HPLC using a mixture of *n*-hexane and acetone (5:1) as the mobile phase to afford the subfractions F1–5. Subfraction F3 was further purified by RP-HPLC using a mixture of methanol (MeOH) and H_2O (97:3, flow rate: 1.0 mL/min) to afford sterols **1** (0.5 mg, t_R = 40 m) and **2** (0.5 mg, t_R = 48 m). Fraction N was separated by NP-HPLC

using a mixture of dichloromethane (CH_2Cl_2) and EtOAc as the mobile phase to afford the subfractions N1–10. Subfraction N9 was further purified by RP-HPLC using a mixture of MeOH and H_2O (9:1, flow rate: 1.0 mL/min) to afford sterol **3** (1.2 mg, t_R = 31 m).

(22R,23R,24R)-5α,8α-Epidioxy-22,23-methylene-24-methylcholest-6,9(11)-dien-3β-ol (**1**): $[\alpha]_D^{25}$ +158 (*c* 0.03, $CHCl_3$); m.p. 218−220 °C; IR (neat) ν_{max} 3445, 1644 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data, see Table 1; ESIMS *m/z* 463 $[M+Na]^+$; HRESIMS: *m/z* 463.3192 (calcd for $C_{29}H_{44}O_3Na$, 463.3188).

(22R,23R,24R)-5α,8α-Epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol (**2**): $[\alpha]_D^{25}$ +20 (*c* 0.02, $CHCl_3$) (Ref. [4], $[\alpha]_D^{26}$ +35 (*c* 0.1, $CHCl_3$)); IR (neat) ν_{max} 3438, 1638 cm^{-1}; ^1H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data were found to be in full agreement with those reported previously [4,18]; ESIMS *m/z* 465 $[M+Na]^+$; HRESIMS: *m/z* 465.3347 (calcd for $C_{29}H_{46}O_3Na$, 465.3344).

24-Methylenecholestane-1α,3β,5α,6β,11α-pentol (**3**): $[\alpha]_D^{25}$ −3 (*c* 0.06, $CHCl_3$) (Ref. [15], $[\alpha]_D^{25}$ −4 (*c* 1.60, $CHCl_3$)); IR (neat) ν_{max} 3380, 1216 cm^{-1}; ^1H (400 MHz, C_5D_5N) and ^{13}C (100 MHz, C_5D_5N) NMR data were found to be in full agreement with those reported previously [15]; ESIMS: *m/z* 487 $[M+Na]^+$; HRESIMS: *m/z* 487.3402 (calcd for $C_{28}H_{48}O_5Na$, 487.3399).

3.4. Cytotoxicity Testing

The cytotoxicity of sterols **1**–**3** was assayed using a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. Cytotoxicity assays were carried out according to previously described procedures [25,26].

4. Conclusions

Steroid metabolites are major constituents of the extracts of *Sinularia* spp. octocorals distributed in the waters off Taiwan [4–13]. Our studies on the chemical constituents of *Sinularia gaweli* have led to the isolation of a new epidioxysterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methyl-cholest-6,9(11)-dien-3β-ol (**1**), along with two known sterols, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3β-ol (**2**) and 24-methylenecholestane-1α,3β,5α,6β,11α-pentol (**3**). Sterol **3** was found to exhibit significant cytotoxicity against HL-60 tumor cells, and this result suggested that sterol **3** is worthy of further biomedical investigation. The soft coral *S. gaweli* has begun to be transplanted to culturing tanks with a flow-through sea water system located in the National Museum of Marine Biology and Aquarium, Taiwan for the extraction of additional natural products in order to establish a stable supply of bioactive material.

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Sample Availability: Samples of the sterols **1**–**3** are available from the authors.